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Title page

Significant Increase in Phenacetin Oxidation upon Leu-382 → Val Substitution in Human Cytochrome P450 1A2

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Running Title Page

Running Title: Increased phenacetin oxidation in P450 1A2 mutants.

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ABBREVIATIONS: P450, cytochrome P450; WT, wild type; NADPH, β-nicotinamide adenine dinucleotide phosphate reduced form; IPTG, isopropyl-β-D-thiogalactopyranoside; ALA, delta-aminolevulinic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; EDTA, ethlenediaminetetraacetic acid; DLPC, dilauroyl-L-3-phosphatidyl-choline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MD, molecular dynamics.

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Abstract

Human cytochrome P450 1A2 is an important drug metabolizing enzyme, similar in sequence to P450 1A1, but with distinct substrate specificity. Previously, we have shown that residue 382 affected 1A1 and 1A2 specificities with alkoxyresorufins. To determine whether this residue is also important for the metabolism of other substrates, we have investigated phenacetin oxidation by single (T124S, T223N, V227G, N312L and L382V) and multiple (L382V/T223N, L382V/N312L, L382V/T223N/N312L and L382V/T124S/N312L) mutants of P450 1A2. The enzymes were expressed in *Escherichia coli* and purified. All P450 1A2 mutants that contained the Leu-382→Val substitution displayed much higher activities than the wild type enzyme, with k_{cat} values 3-fold higher, in contrast to other mutants, for which k_{cat} decreased. Likewise, a significant increase in specificity, expressed as the k_{cat}/K_m ratio, was observed for the mutants containing the L382V substitution. The efficiency of coupling of reducing equivalents to acetaminophen formation was decreased for all single mutants except L382V, for which the coupling increased. This was also observed with multiple 1A2 mutants containing the L382V substitution. Low activities of the four other single mutants were likely due to dramatically increased uncoupling to water. In contrast, the increase in activity of the L382V-containing mutants resulted from decreased water formation. This is consistent with molecular dynamics results, which showed decreased phenacetin mobility leading to increased product formation. The results of these studies confirm the importance of residue 382 in P450 1A2-catalyzed oxidations and demonstrate that a single residue substitution can dramatically affect enzymatic activity.

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Introduction

Cytochromes P450 (P450s) are heme-containing monooxygenase enzymes, which are involved in the metabolism of numerous exogenous and endogenous compounds. P450s are ubiquitous in living organisms, with at least 50 families and 82 subfamilies found in different species. Human P450 1A subfamily has two major isoforms: P450 1A1 and 1A2. P450 1A2, one of the major P450s in the human liver, was first characterized as a phenacetin O-deethylase (Distlerath et al., 1985). Currently, it is estimated that this enzyme metabolizes approximately 11% of all drugs in humans (Shimada et al., 1994). Despite the fact that P450 1A2 participates in the deactivation and detoxification of xenobiotics, the main interest in this enzyme is because of the metabolic activation of a large number of chemical carcinogens (Guengerich and Shimada, 1991; Levis et al., 1994).

In humans, P450 1A2 shares 72% amino acid sequence identity with P450 1A1, but the substrate specificities and inhibitor susceptibilities of these enzymes are different. For example, substrates such as phenacetin and 7-methoxyresorufin are primarily metabolized by P450 1A2 with high catalytic efficiency, while P450 1A1 displays weak capability to oxidize those substrates. On the other hand, 7-ethoxyresorufin is preferentially oxidized by P450 1A1 (Nerurkar et al., 1993; Burke et al., 1994). The structural basis for such functional differences between highly related enzymes can be investigated using a variety of techniques, including molecular modeling and experimental methods, such as site-directed mutagenesis and NMR. Recently, the crystal

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structure of P450 1A2 was solved by X-ray crystallography (Sansen et al., 2007), and thus it provides a practical model for structure-function studies.

Our previous studies on structure-function relationships of P450 1A1 indicated that Val-382 played an important role in binding of alkoxyresorufin substrates (Liu et al., 2003). The sequence alignment between P450 1A1 and 1A2 indicated that five active site residues that are different between these enzymes (Ser-122, Asn-221, Gly-225, Leu3-12 and Val-382 in 1A1 and the corresponding residues in 1A2: Thr-124, Thr-223, Val-227, Asn-312 and Leu-382) might be involved in determining substrate specificity (Liu et al., 2004). This was confirmed by the finding that five reciprocal mutations in P450 1A1 and 1A2 altered enzymatic activity with alkoxyresorufins as substrates. Moreover, mutations at position 382 in both P450 1A1 and 1A2 shifted substrate specificity from one enzyme to another (Liu et al., 2004). Further computational and experimental studies with multiple P450 1A2 mutants confirmed the importance of this residue for alkoxyresorufin oxidation (Tu et al., 2008). Therefore, it would be of interest to examine the effect of these mutations on oxidation of other types of substrates.

In the current study, we chose phenacetin as a substrate. This compound has been used as the most common marker for P450 1A2 activity in the *in vitro* studies of 45% of new drugs by investigators in the pharmaceutical industry (Yuan et al., 2002). The objective of the present study was to investigate whether any reciprocal mutations in P450 1A2 may alter phenacetin oxidation and to examine the potential mechanism(s) that might be involved. A total of five single mutants, and four multiple mutants containing the Leu-382→Val substitution were evaluated using a combination of molecular

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modeling and experimental methods. These included enzyme kinetics and stoichiometry studies, as well as molecular dynamics simulations of phenacetin in the active site of P450 1A2 mutants to facilitate the interpretation of experimental results. This study should provide an increased understanding of the biochemical aspects of substrate specificity in the P450 family of enzymes.

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Materials and Methods

Materials. Phenacetin, acetaminophen, 2-hydroxy acetanilide, sodium dithionite, NADPH, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (ALA), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), dilauroyl-L-3-phosphatidyl choline (DLPC) and phenylmethanesulfonyl fluoride (PMSF) were from Sigma-Aldrich Chemical (St. Louis, MO). Nickel-nitrilotriacetic acid-agarose and a gel extraction kit were purchased from Qiagen (Valencia, CA). Potassium phosphate, EDTA, acetic acid and HPLC grade methanol were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals used were of analytical grade and were obtained from standard commercial sources.

Protein Expression and Purification. The clones of P450 1A1 WT, 1A2 WT and single mutants, T124S, T223N, V227G, N312L and L382V, all of them containing a His-tag for easy purification, were constructed earlier (Liu et al., 2003; 2004). P450 1A2 His-tag multiple mutants, L382V/T223N, L382V/N312L, L382V/T223N/N312L and L382V/T124S/N312L, were also constructed previously (Tu et al., 2008). The P450 enzymes were expressed in *E. coli* DH5α cells and purified essentially as previously described (Liu et al., 2004; Tu et al., 2008). During the purification, the addition of 5 mM caffeine in the purification buffers helped to stabilize P450 1A2 proteins. The substrate caffeine was removed completely from the enzyme preparation during the ultrafiltration stage, as verified by HPLC. Rat cytochrome P450 reductase was expressed in *E. coli* and purified according to an established procedure (Liu et al., 2003). The final

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purity of the enzymes was assessed by SDS-PAGE. Western blots were performed using anti-human P450 1A1/1A2 (Oxford Biomedical, Oxford, MI) and P450 proteins were visualized as described (Kedzie et al., 1991). P450 content was determined by reduced CO/reduced difference spectra (Omura and Sato, 1964) and protein was measured by the method of using Folin phenol reagent (Lowry et al., 1951).

P450 Activity Assay. Phenacetin *O*-dealkylase activities of P450 1A2 WT and mutants were determined by HPLC measurements as described previously (von Moltke et al., 1996), with some modifications. The reaction mixtures contained 0.5 μM P450 1A2, 1 μM P450 reductase, 45 μM DLPC in 100 mM potassium phosphate buffer (pH 7.5). The enzymes and DLPC were pre-incubated for 2 minutes at 37°C before the dilution. For kinetic assays, phenacetin was added at concentrations ranging from 0 to 1000 μM, and the mixture was incubated for another 3 minutes at 37°C. The reaction was initiated by adding NADPH to a final concentration of 1 mM in a total volume of 1 mL, and conducted for 30 minutes. The reaction was terminated by the addition of 5 μL of 60% $HClO_4$ and the reaction mixture was put on ice for 10 minutes. 10 μL of 100 μM 2-hydroxy acetanilide was then added as an internal standard for HPLC determination. The reaction mixture was centrifuged at 10^3g for 5 minutes, and 100 μL of the supernatant was removed and used directly for HPLC analysis. The product acetaminophen was eluted from a C_{18} column (Alltech associates Inc, Deerfield, IL) with a mobile phase of methanol/0.1% acetic acid (30:70, v/v; flow rate 1.5 mL.min^{-1}), and monitored at 254 nm. The product was quantified using acetaminophen standards. The kinetic parameters

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(V_{max} and k_{cat}) were calculated using nonlinear regression with GraphPad Prism software (San Diego, CA).

Binding Constant Determination. Spectral binding constants for phenacetin bound in the active site of P450 1A1 WT and 1A2 enzymes were obtained using difference visible spectroscopy (Modi et al., 1995). Solutions (800 µL) contained 0.5 µM P450 1A2 WT or the mutants in 100 mM phosphate buffer, containing 20% glycerol and 0.1 mM EDTA, pH 7.4. 2 µL of different concentrations of solutions of phenacetin in menthol were added to the sample cuvette, and the same volume of menthol was added to the reference and UV spectra were then recorded. The data was analyzed by nonlinear regression analysis using Microsoft Excel software.

NADPH Oxidation. The rate of NADPH oxidation was determined spectrophotometrically at 340 nm in a cuvette thermostated at 37°C. The reaction mixture was similar to that used for phenacetin assay, and contained 0.5 µM P450 enzyme, 1 µM P450 reductase, 45 µM DLPC and 1 mM phenacetin in a 100 mM potassium phosphate buffer, pH 7.5, in a volume of 980 µL. The reaction was initiated by the addition of 20 µL of 50 mM NADPH. The NADPH oxidation rates were recorded for about 3 minutes at 340 nm from the beginning of the reaction. The molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH at 340 nm was used to obtain oxidation rates in nmol min^{-1} $(nmol\ P450)^{-1}$. Three 50 µL aliquots of the reaction mixture were removed after 1, 2, and 3 minutes, and quenched with 50 µL of 10% CF_3COOH. The triplicate quenched reaction mixture was then used to measure hydrogen peroxide.

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Hydrogen Peroxide Production. The reaction mixtures from NADPH oxidation assay were used to measure the production of hydrogen peroxide (H_2O_2) using the xylenol orange iron (III) assay (Jiang et al., 1990; Fang et al., 1997), with slight modifications. The coloring agent was prepared by mixing 100 volumes of 125 µM xylenol orange in 100 mM sorbitol and 1 volume of 25 mM of fresh ferrous (Fe^{2+}) ammonium sulphate in 2.5 M H_2SO_4. The calibration curve was prepared using the quenched reaction mixture which was supplemented with hydrogen peroxide at concentrations ranging from 0 to 10 µM. The H_2O_2 standard solutions were prepared fresh on the day of the assay by dilution of a 30% H_2O_2 stock solution. The reaction mixture was incubated at room temperature for an hour. Absorbance was recorded using a Beckman spectrophotometer set at 560 nm to obtain the concentration of H_2O_2 produced in nmol min^{-1} (nmol P450)$^{-1}$.

Oxygen Consumption. The reaction was conducted using a Mitocell (Strathkelvin Instruments Limited, Glasgow, U.K.) which was connected to a water bath thermostated at 37°C. The reaction mixture was prepared in a similar way to that for the NADPH oxidation assay. 980 µL of the sample was placed in the chamber of the Mitocell and once a steady baseline was established, the reaction was initiated by the addition of 20 µL NADPH. The oxygen consumption was recorded over 5 min as µmol L^{-1} $hour^{-1}$, which was then converted to nmol min^{-1} (nmol P450)$^{-1}$.

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Molecular Modeling Methods: General. Molecular modeling simulations were conducted using a Silicon Graphics Octane workstation with Insight II software (Accelrys, San Diego, CA). The crystal structure of P450 1A2 (pdb code: 2hi4) was obtained courtesy of Dr. Eric F. Johnson, The Scripps Research Institute, La Jolla, CA (Sansen et al., 2007). The heme cofactor was removed and replaced with the oxoheme cofactor. Substrate phenacetin was constructed with Insight II/Builder module, and optimized. The models of P450 1A2 single and multiple mutants were constructed from the crystal structure of 1A2 WT by the replacement of selected amino acid(s) and further refinement of the structures according to the previously established procedure (Liu et al., 2003; 2004, Tu et al., 2008). Molecular dynamics (MD) simulations and energy minimization were carried out using the Insight II/Discover module with the consistent valence force field (CVFF) supplemented with parameters for heme and ferryl oxygen, as described earlier (Paulsen and Ornstein, 1991; 1992). The non-bond cutoff was 16 Å, and all other parameters were set at their default values. Structural refinement of P450 1A2 WT and mutants involved 1000 steps of minimization using steepest descent gradient followed by 10 ps MD and then another 1000 steps of steepest descent minimization. The optimized structures were used for the subsequent docking studies.

Docking of Phenacetin into the Active Site of P450 1A2 WT and Mutants. Initially, phenacetin was manually placed into the active site of P450 1A2 WT and the mutants on the distal side of the oxoheme. Docking of phenacetin was performed with Insight II/Affinity module using default parameters, as described previously (Liu et al., 2004; Ericksen and Szklarz, 2005; Tu et al., 2008). Residues within 10 Å of the initial

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phenacetin position comprised the flexible region of the receptor (P450 1A2 WT and mutants) during all docking runs. The Affinity docking method utilizes both the Monte Carlo (MC) search technique and simulated annealing (SA) approach followed by the minimization protocol to generate low-energy substrate binding orientations. A distance-dependent dielectric constant was applied to simulate charge screening by water molecules. Ten lowest-energy phenacetin binding orientations obtained from Affinity docking were selected for further analysis.

Molecular Dynamics (MD) Simulations of Enzyme-Substrate Complexes. MD simulations were performed to investigate phenacetin mobility in the active site and the effect of mutations on substrate orientation. The starting configuration for MD simulations chosen from Affinity docking represented the productive binding orientation of phenacetin leading to its *O*-dealkylation, and had the lowest potential energy rank. The MD simulations of each phenacetin-enzyme complex were performed at 310 K *in vacuo* essentially as described earlier (Liu et al., 2004; Tu et al., 2008). The substrate, heme and protein residues within 10 Å from the initial substrate position were flexible without any restraints, while the remainder of the protein was fixed. A distance-dependent dielectric constant was used to simulate aqueous environment, and the non-bond cutoff distance was 16 Å. After 5 ps MD equilibration phase, the MD simulations were continued for 100 ps, and 400 frames obtained every 250 fs were extracted to record the snapshots of each enzyme-substrate complex.

Additionally, to evaluate the effect of explicit solvent on phenacetin dynamics in the active site, we performed 100 ps MD simulations on solvated enzyme-substrate

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complexes using a similar protocol. The enzymes chosen were P450 1A2 WT and the L382V mutant. The enzyme-substrate complexes were solvated using crystallographic water molecules from P450 1A2 crystal structure and optimized prior to MD with 1000 steps of steepest descent minimization using a non-bond cutoff of 16 Å and dielectric constant of 1. Similar to previous MD simulations, only protein residues, heme, substrate and solvent within a 10 Å radius of the initial substrate position were permitted to move, while the remainder of the protein and other water molecules were fixed. This insures that none of the moving solvent molecules escape from the vicinity of the active site. In contrast to previous MD simulations, the flexible region also included 21 water molecules surrounding phenacetin. For the aqueous simulations, the dielectric constant was set to 1. All other parameters were the same as for the previous enzyme-substrate simulations without explicit solvent present.

All MD trajectories were examined using Insight II/Analysis module. To score the likelihood of hydroxylation, each sampled frame of a given enzyme-substrate complex was evaluated using the following geometric criterion: $r \leq 3.5$ Å and $\theta \geq 120^\circ$, where r represents the distance between the ferryl oxygen and the hydrogen of the substrate to be abstracted; θ represents the angle between ferryl oxygen, the hydrogen atom to be abstracted, and the carbon at the oxidation site, as described previously (Ericksen and Szklarz, 2005; Tu et al., 2008). Trajectory data from 100 ps MD simulations were extracted and graphed using Microsoft Excel. The MD frames where the geometric criterion, $r \leq 3.5$ Å and $\theta \geq 120^\circ$, was satisfied, were counted as hits. The number of hits is a useful indicator of whether a P450 1A2-mediated phenacetin *O*-deethylation occurred.

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Results

Kinetics of Phenacetin *O*-Deethylation by P450 1A2 WT and Mutants. P450 1A2 enzymes were expressed in *E. coli* and purified. The overall yield of the procedure was about 20-40%, similar to that reported previously (Liu et al., 2004; Tu et al., 2008). The purity of P450 1A2 WT and mutants verified by SDS-PAGE and Western blots indicated that they were at least 95% pure. The spectrum of the Fe^{II}-CO complex exhibited a characteristic peak at 450 nm, with little or no P420 formation. The holoenzyme content of the enzymes was usually in the range of 40-60%, as previously observed in our laboratory.

Kinetic parameters, k_{cat}, K_m and substrate specificity (k_{cat}/K_m), were determined for purified P450 1A2 WT and mutants using a range of substrate phenacetin concentrations. Phenacetin undergoes *O*-deethylation to form acetaminophen as the main product of the reaction. Kinetic parameters for P450 1A2 WT and mutants are shown in Table 1. Compared to P450 1A2 WT, the mutations affected both k_{cat} and K_m. Interestingly, the P450 1A2 L382V mutant and multiple mutants containing the Leu-382→Val mutation displayed 2-fold or 3-fold higher k_{cat} than the wild type enzyme. Four other single mutants, namely T124S, T223N, V227G, and N312L, exhibited much lower k_{cat} than the WT.

As shown in Table 1, the K_m values for 1A2 WT and mutants varied greatly. Generally, the K_m values for all the mutants were more than 50% lower than for the wild type (~60 μM). The lowest values of K_m were observed for several mutants containing the L382V substitution, including L382V (~6 μM), L382V/T223N (~8 μM), and

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L382V/T124S/N312L (~10 μM), which suggests that the Leu-382→Val mutation significantly increased the binding affinity of the enzyme for phenacetin. The substrate specificities of the L382V mutant and multiple mutants containing the Leu-382→Val mutation, expressed as k_{cat}/K_m, were at least 5-fold higher than that of the WT (Table 1). In particular, the relative substrate specificities of L382V and L382V/T223N mutants were over 20-fold higher, which is extremely high.

Incidentally, phenacetin is less efficiently metabolized by P450 1A1 WT, (k_{cat} 0.5 min^{-1} and K_M 66 μM), with k_{cat} close to one third of the value observed with P450 1A2 WT (see Table 1) and the k_{cat}/K_m ratio lower than 0.01.

Phenacetin binding constants were also determined for P450 1A2 WT and some mutants. P450 1A2 WT and the N312L mutant showed similar phenacetin binding, with binding constants of 17.1 μM and 10.2 μM, respectively. In contrast, the L382V and the L282V/N312L mutants displayed much lower values for binding constants, namely 0.7 μM and 3.5 μM, indicating tighter substrate binding. Interestingly, P450 1A1 WT exhibited much weaker binding, with a binding constant of 57 μM.

Stoichiometry of Phenacetin *O*-Deethylation. To assess whether the Leu-382→Val mutation affected P450 1A2 coupling efficiency of reducing equivalents to acetaminophen formation, stoichiometry experiments were conducted. The rates of NADPH oxidation, hydrogen consumption, product formation, hydrogen peroxide and excess water production for phenacetin oxidation by P450 1A2 WT and mutants are shown in Table 2. The excess water formation was calculated from the difference between the rates of NADPH oxidation, and rates of hydrogen peroxide and product

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formation (Excess H_2O = NADPH – H_2O_2 – product). The amount of water was also obtained from the difference between the rate of oxygen consumption and rates of hydrogen peroxide plus product formation ($H_2O = 2(O_2 - H_2O_2$ – product)), as reported by others (Fang et al., 1997), giving the values which were very similar (within 5%) to those derived from the previous equation (data not shown). The coincubation of phenacetin with P450 1A2 L382V and three multiple mutants, L382V/T223N, L382V/N312L and L382V/T223N/N312L, resulted in consumption of both NADPH and oxygen by the mutants at rates ~2-fold greater than those with the wild type enzyme. Likewise, the formation of product acetaminophen as well as byproducts such as hydrogen peroxide and water, were 2-3-times higher in the case of these mutants. The exception was the L382V/T124S/N312L mutant, which seemed to utilize NADPH at a rate similar to the WT enzyme, but displayed increased consumption of oxygen, along with increased product and hydrogen peroxide formation. On the other hand, four single mutants, T124S, T223N, V227G and N312L, are similar or less efficient than the WT with respect to NADPH oxidation, oxygen consumption, hydrogen peroxide, and water production, but exhibit a substantial decrease in product formation.

Table 3 presents the effects of mutations on the coupling efficiency of P450 1A2, both overall and at specific P450 uncoupling branching points. The ratios of product formation to NADPH oxidation, accounting for the overall efficiency of P450 1A2 coupling of reducing equivalents to product, were higher for L382V and multiple mutants containing the L382V substitution than for the WT. In contrast, other single mutants, T124S, T223N, V227G and N312L, exhibited significantly decreased coupling efficiencies compared to the WT enzyme (20-30% of WT). Interestingly, all enzymes

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displayed similar ratios of H_2O_2 production to O_2 consumption, which suggests that the mutations had no effect on uncoupling at the first and second branching points of the P450 cycle. On the other hand, significant differences between the enzymes were observed with respect to the H_2O: product ratios used to measure uncoupling at the third branching point. Thus, for the L382V mutant and multiple mutants containing the Leu-382→Val substitution, these ratios were generally lower than that for the WT, while for the other single mutants, these ratios were 3-fold higher. Therefore, low activities of the four single mutants were likely due to dramatically increased uncoupling to water, whereas the increase in activity in the L382V-containing mutants resulted from decreased water formation.

Molecular Modeling Analyses. Using the crystal structure of P450 1A2, molecular modeling studies have been conducted to understand the effects of single and multiple mutations on enzyme-substrate interactions and substrate mobility, and to explain the alterations of catalytic efficiency. Figure 1 depicts binding orientations of phenacetin within the active sites of P450 1A2 WT and the L382V mutant. In general, the binding orientation of phenacetin in both enzymes was very similar. However, the replacement of Leu-382 by a smaller Val increased the volume of the active site and allowed the hydrogens at the oxidation site of phenacetin to approach closer to the ferryl oxygen of the heme, which facilities hydrogen abstraction. The average distance between the hydrogens at the oxidation site of phenacetin and the ferryl oxygen of the L382V mutant was 3.1 Å, compared to 3.7 Å for the WT enzyme. Similar results were also obtained for the L382V/T223N and L382V/T223N/N312L mutants. In contrast, both hydrogens at the

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oxidation site of phenacetin were much farther away from the ferryl oxygen in the active sites of T124S, T223N, V227G, and N312L mutants (data not shown). These findings correlate well with the results of kinetic and stoichiometric studies described above, and suggest that the substitution of Leu-382 with Val not only increases catalytic efficiency (k_{cat}) of P450 1A2 but also decreases K_m and enzyme uncoupling.

In order to examine the tendency of phenacetin to remain in the productive binding orientation in the active site of the enzyme, 100 ps of molecular dynamics (MD) simulations were performed, as described in *Materials and Methods*. After the 5 ps MD equilibration phase, the energy of the system remained constant throughout the simulations. The mobility of the substrate varied with the mutant: we observed fairly low mobility for the WT enzyme, L382V, T124S and L382V/T223N/N312L mutants, with RMSD from the initial substrate orientation of 1-2 Å, moderate mobility for V227L and N312L mutants with RMSD of 2-4 Å, and a high phenacetin mobility in the case of T223N and L382V/T223N mutants (RMSD > 4 Å).

The productive binding orientations of phenacetin were determined using geometric parameters, distance r and angle θ, as described in *Materials and Methods*. These parameters were then plotted based on 400 recorded snapshots for each enzyme-substrate complex. The representative plots, those for P450 1A2 WT, N312L, L382V, and L382V/T223N mutants, showing the ensembles of substrate orientations, are presented in Fig. 2. The region where the geometric criterion ($r \leq 3.5$ Å and $\theta \geq 120^\circ$) is satisfied is gray, and the points (or frames) located within represent all snapshots of each enzyme-substrate complex where phenacetin was bound in the productive binding orientation. The distribution of the MD frames for each enzyme-substrate complex

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displayed varied. Overall, phenacetin showed higher occupancy within a productive binding region (counted as hits) in the L382V and L382V/T223N mutants than in the WT. Similar results were also seen in the case of other multiple mutants containing L382V (data not shown), while few hits if any were observed for the remaining four single mutants, as shown for the N312L mutant (Fig. 2b). A quantitative analysis of the hits for each enzyme-substrate complex revealed that the number of hits for L382V, L382V/T223N, and L382V/T223N/N312L mutants were 2-fold, 4-fold and 5-fold higher, respectively, than that for the WT (Table 4). In contrast, zero or few hits were obtained for T124S, T223N, V227G, and N312L mutants during 100 ps dynamics. It is possible that some hits might be recorded during a longer simulation time. Overall, the results of MD simulations are, like those from docking experiments, consistent with the kinetic and stoichimetric analyses of P450 1A2 WT and mutants.

A possible drawback of the simulations described above might have been the utilization of the distance-dependent dielectric constant instead of explicit solvent. Therefore, we have also conducted MD simulations of phenacetin docked in the active site of P450 1A2 WT and the L382V mutant with the solvent molecules present and the dielectric constant of 1. In the presence of water, 70 hits were recorded for the WT enzyme and 144 hits for the L382V mutant. Thus, in both cases, the percentage of hits increased less than 10% and to a very similar extent (9.4% for the WT and 7.5% for the mutant) compared to the results from MD simulations without water (see Table 4). This indicates that, although some changes may be observed with solvent present, simulations with distance-dependent dielectric provide a reasonable approach to explain the observed experimental findings.

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Discussion

Our previous studies indicated that residue 382 plays an important role in controlling the specificity of alkoxyresorufin *O*-dealkylation by P450 1A1 and 1A2 (Liu et al., 2003; 2004; Tu et al., 2008). Since phenacetin is another important probe substrate for P450 1A2, the studies to address the effects of reciprocal mutations, particularly Leu-382→Val, on phenacetin oxidation, may broaden our understanding of the role of this residue in substrate specificity. In the present study, a total of 9 single and multiple mutants were investigated using a battery of complementary approaches, such as kinetic assays, stoichiometry measurements and molecular modeling methods. The results demonstrated that the substitution of Leu-382 with Val resulted in a significant increase in catalytic activity and substrate specificity of P450 1A2. Interestingly, all multiple mutants that contained this substitution displayed very similar kinetics, stoichiometry, and dynamic mobility as the single L382V mutant. Thus, the presence of this single residue was critical for dramatically improving the efficiency of phenacetin oxidation by P450 1A2.

To date, many residues of P450 1A2 have been identified that may play a role in enzyme-ligand interactions by site-directed mutagenesis and/or molecular modeling studies (Yun et al., 2000; Liu et al., 2004; Zhou et al., 2009). In the case of single mutants, most of the mutations, including T124S, T223N, V227G, and N312L in the present study, resulted in decreased catalytic activity and substrate specificity compared to the wild-type enzyme (Parikh et al., 1999; Liu et al., 2004). A number of P450 1A2 mutants obtained from random mutagenesis showed increased catalytic activities and substrate specificities toward phenacetin (Parikh et al., 1999), but none of them was as

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highly active as the L382V mutant reported in this study (Table 1). It is worth mentioning that the kinetic parameters for phenacetin *O*-dealkylation by P450 1A2 WT determined in the present investigation were very similar to those reported by Parikh et al. (1999). Although the Leu-382→Val mutation dramatically increased oxidation of phenacetin, it led to a significant decrease in 7-methoxyresorufin *O*-dealkylation (Liu et al., 2004). Thus, the functional effect of residue substitution appears to be dependent on the substrate (Zhou et al., 2009).

Moreover, the binding constants determined for P450 1A2 WT and mutants showed that the substitution of Leu-382 with Val leads to tighter phenacetin binding in the active site of the L382V-containing mutants, consistent with enzyme kinetics results. This may increase phenacetin residence time resulting in higher activity.

In order to better explain the effects of mutations on catalytic activity and substrate specificity of P450 1A2, stoichiometry studies were performed. The coupling efficiencies of different mutants, expressed in terms of product:NADPH, H_2O_2:O_2, and H_2O:product ratios, were compared to those of WT. A similar approach has been used by Fang et al. (1997) and Kobayashi et al. (1998) to evaluate coupling efficiencies of P450 2B1 mutants. Frequently, the mutation decreases the coupling efficiency of the P450, as observed with P450 2B1 (Fang et al., 1997; Kobayashi et al., 1998) and P450cam (French et al., 2002), but the effect depends upon the substrate. Previously studied P450 1A2 mutants showed only a small increase in coupling efficiency with phenacetin as a substrate (Yun et al., 2000), in contrast to our results with the L382V-containing mutants. In the present studies, the L382V mutant and multiple mutants containing the Leu382→Val substitution were similar or more efficient at coupling

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reducing equivalents to acetaminophen formation than the WT (Table 3). In general, the increased ratios of product:NADPH for the L382V mutant and multiple mutants and the decreased ratios for T124S, T223N, V227G, and N312L mutants were in agreement with the kinetic data regarding phenacetin turnover rates. Although no significant changes of uncoupling to H_2O_2 at the first and the second branching points were observed in all the mutants, those which contained the Leu-382→Val substitution showed less uncoupling to water (decreased H_2O:product ratio, Table 3). Thus, it seems reasonable to suggest that the substitution of Leu-382 with Val in P450 1A2 yields the mutants that utilize NADPH and O_2 more efficiently to oxidize phenacetin to products and display less uncoupling to water, so that the overall coupling efficiency of the enzyme increases, which is consistent with enzyme kinetics results (Table 1).

Molecular modeling studies provide another possible explanation of the effects of mutations on phenacetin specificity, as indicated by changes in kinetic parameters. For these simulations, we used the X-ray structure of P450 1A2, and the structures of the mutants were derived from the crystal. As reported by Sansen et al. (2007), this P450 1A2 structure has a closed compact active site, without clear solvent or substrate access channels, with a relatively small volume of the cavity, estimated at 375 Å^3. The active site of 1A2 is about 44 % larger than that of P450 2A6 (260 Å^3) and significantly smaller than that of P450 3A4 (1385 Å^3) (Sansen et al., 2007). Consequently, only planar compounds, such as α-naphthoflavone (ANF), and typical P450 1A2 substrates such as phenacetin, 7-ethoxyresorfin, caffeine, tacrine or theophylline can be fitted well with the narrow and flat active site cavity of enzyme. Leu-382 of P450 1A2 is a critical residue located close to the heme iron and its replacement with a smaller Val increases the

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volume of the active site near heme. This allows phenacetin to move closer to heme, so that one of the hydrogens at the oxidation site is within a hydrogen-bonding distance from the ferryl oxygen (Fig. 1), which promotes hydrogen abstraction. This is consistent with the MD results (Table 4, Fig. 2). Consequently, the movement of phenacetin closer to the ferryl oxygen in the L382V mutants helps to explain not only the substantial increase in k_{cat} and a decrease in K_m for the production of acetaminophen (Table 1), but also more efficient coupling of the P450 reaction cycle with less water formation (Table 3).

Molecular dynamics simulations similar to those described in this work have been successfully used in our previous studies for fairly rapid predictions or interpretation of experimental results (Ericksen and Szklarz, 2005; Tu et al., 2008). The present studies suggest that the use of the distance-dependent dielectric constant can be a reasonable substitute for the presence of explicit water molecules. We have observed only a small increase in the percentage of hits (less than 10%) from MD simulations with water. These conclusions may be further verified by more extensive MD simulations of the complete protein-substrate complexes on nanosecond time-scales. Recently, longer 2 ns MD simulations have been successfully used to predict the effect of mutations on the catalytic efficiency of P450 2B6 (Nguyen et al., 2008).

In summary, our results demonstrate that the substitution of Leu-382 with Val in P450 1A2 can alter the binding orientation of phenacetin within the active site of the enzyme, so that the site of metabolism moves closer to the heme iron. This provides a good mechanistic explanation for the increased catalytic efficiency as well as coupling efficiency of phenacetin *O*-dealkylation in 1A2 mutants containing the Leu-382→Val

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substitution. The current studies also demonstrate that a combination of several experimental approaches with molecular modeling methods can improve our understanding of P450 catalysis. These different methodologies complement each other well to offer a mechanistic interpretation of P450 function on a molecular level.

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Footnotes

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Legends for Figures

FIGURE 1. Binding orientation of phenacetin within the active site of P450 1A2 WT (A) and the L382V mutant (B). The protein backbone is depicted as a blue ribbon, the side chain of residue 382 is green, with van der Waals surface displayed, heme is red, and phenacetin is yellow, with hydrogens at the oxidation site shown in red. The distance between the hydrogen to be abstracted and the ferryl oxygen (marked with a black line) is 3.6 Å in the WT and 2.9 Å in the L382V mutant.

FIGURE 2. Ensembles of substrate orientations obtained from 100 ps MD simulations of phenacetin-enzyme complexes described by geometric parameters, distance r and angle θ. H_1 in $–OCH_2–$ group of phenacetin is blue and H_2 is red. Grey regions, where the criterion ($r \leq 3.5$ Å and $\theta \geq 120^\circ$) is satisfied, represent productive binding orientations of phenacetin within the active site. P450 1A2 enzymes were: WT (A), N312L mutant (B), L382V mutant (C) and L382V/T223N mutant (D).

TABLE 1

Kinetic parameters for phenacetin O-deethylation by purified P450 1A2 WT and mutants

P450 1A2	Phenacetin *O*-deethylation			
	k_{cat} (min^{-1})[a]	K_m (μM)[a]	k_{cat}/K_m ($\mu M^{-1}\ min^{-1}$)[b]	$[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{WT}]$
WT	1.28±0.08	59.70±1.82	0.02±0.01	1.00
T124S	0.29±0.03	28.90±1.10	0.01±0.01	0.47
T223N	0.30±0.01	26.99±0.01	0.01±0.01	0.52
V227G	0.38±0.02	32.36±2.93	0.01±0.01	0.55
N312L	0.31±0.01	25.26±0.01	0.01±0.01	0.57
L382V	3.03±0.06	5.75±0.57	0.53±0.04	24.77
L382V/T223N	3.91±0.10	7.77±0.80	0.51±0.06	23.65
L382V/N312L	3.05±0.15	16.14±2.05	0.19±0.02	8.87
L382V/T223N/N312L	3.67±0.10	32.42±11.31	0.12±0.04	5.61
L382V/T124S/N312L	2.81±0.03	10.13±2.40	0.28±0.07	13.34

[a] Data are means of triplicate determinations.

[b] k_{cat}/K_m represents substrate specificity.

TABLE 2

Rates (nmol min^{-1} (nmol P450^{-1})) determined for phenacetin metabolism by P450 1A2 wild-type and mutants[a]

P450 1A2	NADPH oxidized	O_2 consumed	Product formed	H_2O_2 produced	Excess H_2O[b]
WT	43±4	22.7±0.9	1.28±0.08	14±2	15±2
T124S	27±2	14.8±0.4	0.29±0.03	10±0	9±1
T223N	41±3	17.3±0.7	0.30±0.00	10±1	14±2
V227G	40±3	16.9±0.5	0.38±0.02	9±1	15±2
N312L	32±2	18.3±0.3	0.31±0.00	13±2	10±1
L382V	84±6	47.0±1.3	3.03±0.06	29±3	30±4
L382V/T223N	98±7	57.9±2.5	3.91±0.10	38±3	32±5
L382V/N312L	70±4	41.0±2.0	3.05±0.15	29±3	18±3
L382V/T223N/N312L	72±5	41.7±1.1	2.18±0.03	28±3	23±3
L382V/T124S/N312L	42±3	34.2±0.9	3.67±0.10	26±2	9±2

[a]Data are means of triplicate determinations.

[b]Excess water (H_2O) was calculated from the equation: H_2O= NADPH – H_2O_2 – product

TABLE 3

Effect of mutations on coupling efficiency of P450 1A2 at different branching points of P450 cycle[a]

P450 1A2	Product/NADPH[b]	H_2O_2/O_2[c]	H_2O/Product[d]
WT	0.030	0.62	11.72
T124S	0.011	0.68	31.03
T223N	0.007	0.58	46.67
V227G	0.010	0.53	39.47
N312L	0.010	0.71	32.26
L382V	0.036	0.62	9.9
L382V/T223N	0.040	0.66	8.18
L382V/N312L	0.044	0.71	5.9
L382V/T223N/N312L	0.030	0.67	10.55
L382V/T124S/N312L	0.087	0.76	2.45

[a]Ratios were calculated from parameters in Table 2.

[b]Efficiency of coupling reducing equivalents to product.

[c]Effect on uncoupling at first or second branch point.

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[d]Effect on uncoupling at third branch point.

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TABLE 4

Geometric analysis of molecular dynamics results for P450 1A2 WT and mutants

Hits represent the MD frames where phenacetin was in the productive binding orientation, as evaluated by the geometric criterion: $r \leq 3.5$ Å & $\theta \geq 120$ ˚.

P450 1A2	Number of Hits
WT	64
T124S	0
T223N	0
V227G	4
N312L	0
L382V	134
L382V/T223N	252
L382V/T223N/N312L	308

Figure 1

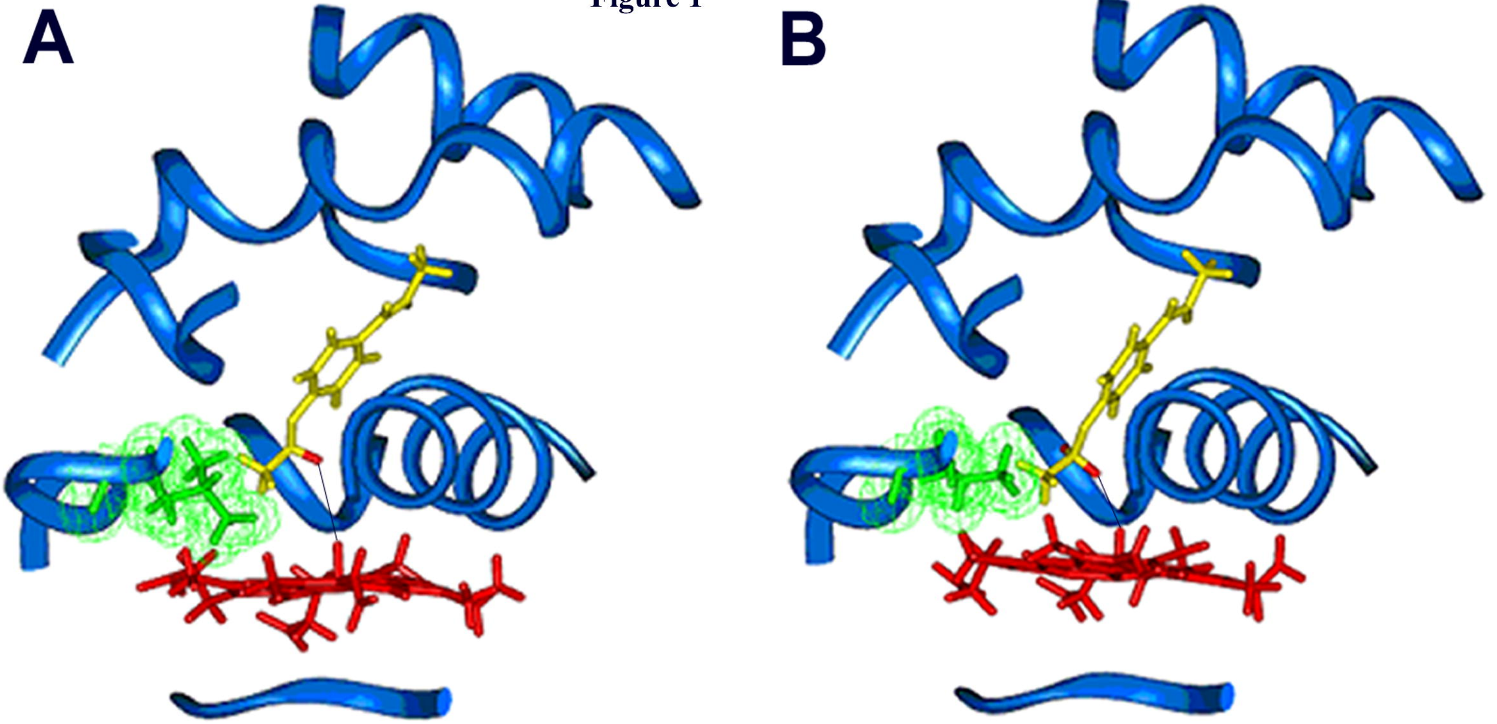
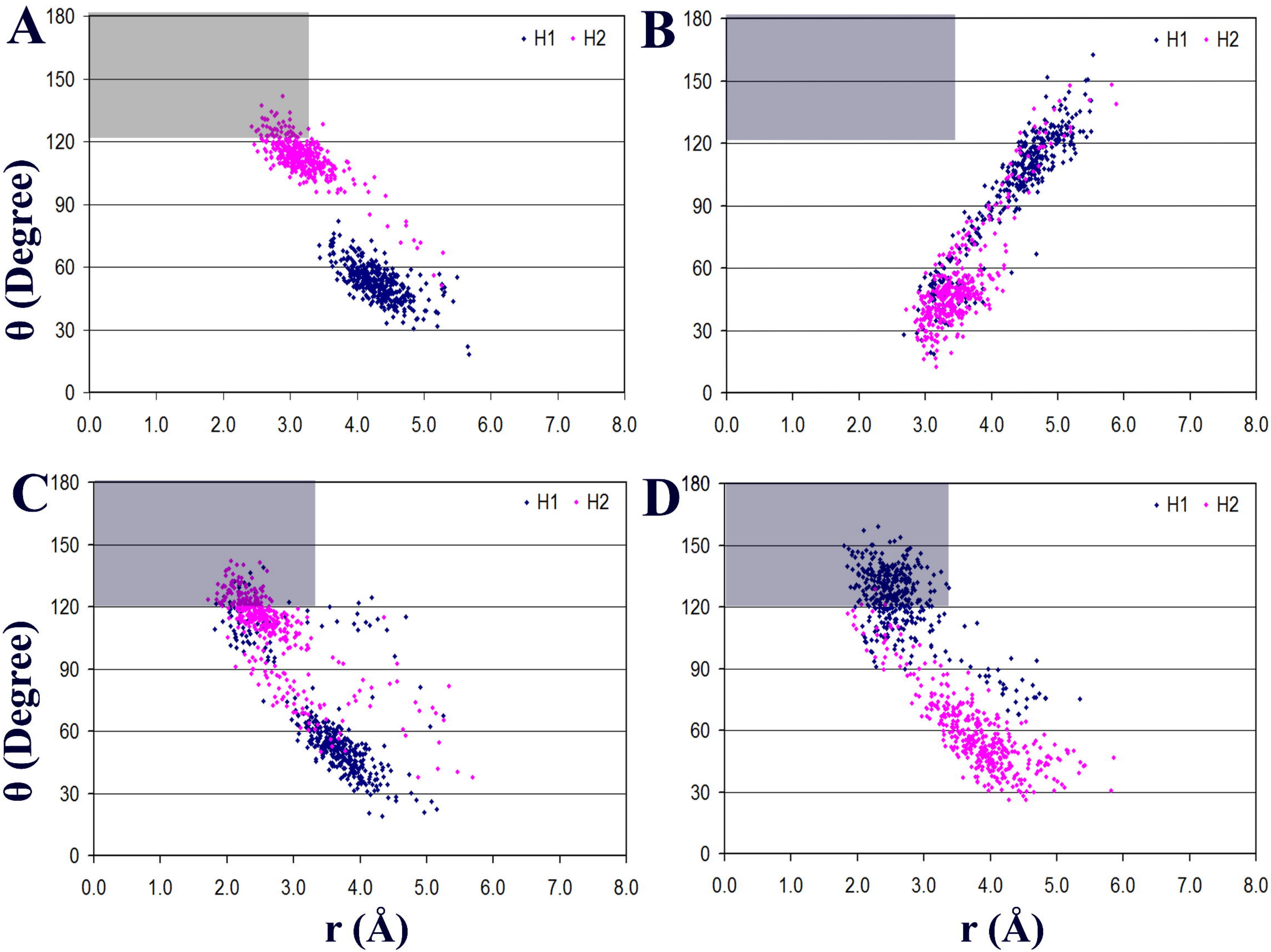


Figure 2


A
B
C
D
H1
H2
θ (Degree)
r (Å)
180
150
120
90
60
30
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0